LETTERS

Role of PIP_2 in regulating *versus* modulating Ca^{2+} channel activity

We would like to comment on an article by Gamper & Shapiro (2007) recently published in *The Journal of Physiology* entitled Target-specific PIP_2 signalling: How might it work? In this paper, they contend that, 'there is wide agreement that the $G_{q/11}$-coupled muscarinic modulation of both N-type Ca^{2+} and M-type K^+ channels is mediated by receptor-induced depletion of PIP_2'. We disagree with Gamper and Shapiro's portrayal of widespread acceptance of this model for Ca^{2+} channel modulation by G_q-coupled receptors. In this paradigm, loss of PIP_2 association with Ca^{2+} channels is necessary and sufficient for current inhibition (Wu *et al.* 2002; Gamper *et al.* 2004; Michailidis *et al.* 2007). Activation of phospholipase C (PLC) by G_q-coupled receptors substantially lowers free PIP_2 levels in the plasma membrane. As a consequence, when constitutively bound PIP_2 dissociates and diffuses away from the channel rather than rebinding, channel activity is inhibited (Gamper *et al.* 2004). No other signalling events, e.g. kinases, phosphatases or additional lipases, participate in channel inhibition. This hypothesis arose from a similar model for M-current modulation by M_1Rs, first proposed by Suh & Hille (2002) and later embraced by several labs examining Ca^{2+} current modulation by G_q-coupled receptors (Wu *et al.* 2002; Gamper *et al.* 2004; Robbins *et al.* 2006).

We have offered an alternative hypothesis to Gamper *et al.* (2004) as to the signal cascade that confers Ca^{2+} current inhibition. Studies in our lab indicate that in addition to PLC, group IVa phospholipase A_2 ($cPLA_2$) activity is required in order to observe modulation of L- and N-type Ca^{2+} currents. Our conclusion is based on the following specific findings. (1) Arachidonic acid (AA) mimics the actions of M_1R agonists in inhibiting channel activity (Liu & Rittenhouse, 2000, 2003*b*; Barrett *et al.* 2001; Liu *et al.* 2001, 2006). (2) The presence of bovine serum albumin (BSA), either in the pipette or in the bath solution, minimizes L- and N-current inhibition by Oxo-M (Liu & Rittenhouse, 2003*b*; Liu *et al.* 2006). Moreover, AA rather than a metabolite, mediates N-current modulation since blocking AA's metabolism has no effect on exogenous AA or Oxo-M's ability to inhibit current (Barrett *et al.* 2001; Liu *et al.* 2001; Liu & Rittenhouse, 2003*b*). (3) Antagonizing PLA_2 with oleyloxyethyl phosphorylcholine (OPC) minimizes inhibition of both L- and N-currents (Liu & Rittenhouse, 2003*a*,*b*; Liu *et al.* 2004, 2006). Our OPC data contrast findings from Gamper *et al.* (2004) who reported no effect of OPC on N-current inhibition by Oxo-M. Similarly, Bannister *et al.* (2002) found that the PLA_2 antagonist quinacrine had no effect on M_1R inhibition of recombinant L-current ($Ca_V1.2$). However, both studies failed to provide controls demonstrating that the PLA_2 antagonist was effectively blocking the enzyme, so that the respective conclusions appear premature. (4) Using antibodies as functional antagonists, we found that dialysing cells with antibodies to $cPLA_2$, but not to $sPLA_2$ or non-immunized antibody minimized L-current inhibition (Liu & Rittenhouse, 2003*a*; Liu *et al.* 2004, 2006). (5) Using a genetic approach we found that neurons from mice deficient in $cPLA_2$ ($cPLA_2^{-\backslash-}$) exhibited minimal L-current inhibition by Oxo-M (Liu *et al.* 2006). No significant differences in control current amplitude or magnitude of current inhibition by AA was observed between $cPLA_2^{+/+}$ *versus* $cPLA_2^{-/-}$ neurons, indicating normal channel activity in $cPLA_2^{-/-}$ neurons. Moreover, M-current inhibition by Oxo-M remained normal, indicating no change in M_1R, G_q, or PLC functioning in $cPLA_2^{-/-}$ neurons. However, $cPLA_2^{-/-}$ neurons exhibited decreased fatty acid release following exposure to Oxo-M compared to wild-type neurons, consistent with a requirement for $cPLA_2$-dependent increases in free fatty acid levels in order to observe L- and N-current inhibition. (6) Using BSA as an AA scavenger to limit free fatty acid levels antagonized L- and N-current inhibition (Liu & Rittenhouse, 2003*b*; Liu *et al.* 2006). Taken together our findings indicate that lipid products downstream of PIP_2 are required for Ca^{2+} current modulation, whereas M-current inhibition appears to occur with PIP_2 breakdown by PLC. Most critically, the studies with $cPLA_2^{-/-}$ neurons document that PLC activity alone is insufficient to mediate Ca^{2+} current inhibition.

How to reconcile our previous findings that a fatty acid (probably AA) mediates Ca^{2+} current inhibition by M_1R signalling with the PIP_2 model remains unresolved (Liu & Rittenhouse, 2003*b*; Liu *et al.* 2004, 2006; Michailidis *et al.* 2007). Resolution will come with additional controls and experiments. For example the PIP_2 analogue DiC8-PIP_2 appears to minimize Ca^{2+} current inhibition; however, this analogue of PIP_2 does not contain the normal fatty acid chains associated with PIP_2, e.g. AA and stearic acid. It is possible that diC8-PIP_2 acts as a substrate competitor with other phospholipids for $cPLA_2$ decreasing the AA liberated and minimizing Ca^{2+} channel inhibition. Similarly, whether application of exogenous phospholipids swells membranes to such an extent that M_1R signalling no longer functions needs to be tested and resolved. Most critical is the need to distinguish between questions that test how PIP_2 functions as a regulator of channel activity *versus* its role in Ca^{2+} channel modulation by specific G_q-coupled receptors. For example, dephosphorylating PIP_2 may cause PIP_2 to dissociate from Ca^{2+} channels and lower activity; however, during muscarinic signalling, this mechanism may play no role in decreasing current amplitude. In recent work palmitoylated charged peptides, that sequester PIP_2, were dialysed into neurons, decreasing both M- and N-current amplitudes. However, low concentrations of peptide, which only minimally decreased basal current amplitude, disrupted M- but not N-current modulation by M_1Rs (Robbins *et al.* 2006). The different results underscore the notion that specific experiments testing roles in modulation as well as regulation are needed in order to properly define the function of PIP_2 in ion channel regulation. Thus experiments outlined by Gamper & Shapiro (2007) that test how plasma membrane PIP_2 levels are regulated contribute to our understanding of PIP_2's functioning, but not necessarily its role in modulating Ca^{2+} channel activity. Thus, we strongly encourage caution when using these experiments as evidence that a simple dissociation of PIP_2 from Ca^{2+} channels explains how M_1Rs inhibit Ca^{2+} channel activity. Our model provides additional levels of control allowing more independent regulation of specific ion channel activity by M_1R signalling.

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